IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Kuliopulos and Covic		
SERIAL NUMBER:	09/841,091	EXAMINER:	Sandra L. Wegert
FILING DATE:	April 23, 2001	ART UNIT:	1647
FOR:	G PROTEIN COUPLED RECEPTOR (GPCR) AGONISTS AND ANTAGONISTS AND METHODS OF ACTIVATING AND INHIBITING GPCR USING THE SAME		

Commissioner for Patents
P.O. Box 1450
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DECLARATION UNDER 37 C.F.R. § 1.132

I, Athan Kuliopulos hereby declare and state as follows:

1. I am employed by New England Medical Center Hospitals, Inc., the assignee of this application. My title is Associate Professor of Medicine and Biochemistry at Tufts University School of Medicine, and I am a member of the Molecular Oncology Research Institute. I received my M.D./Ph.D. from Johns Hopkins University.

2. I have read, and am familiar with, the contents the Office Action mailed on September 30, 2003 for United States Patent Application entitled "G Protein Coupled Receptor (GPCR) Agonists and Antagonists and Methods of Activating and Inhibiting GPCR Using the Same" U. S. Serial Number 09/841,091, which was filed April 23, 2001. In particular, I understand the Examiner's grounds for rejecting the claims.

3. In addition to the data described in the specification as filed, I have made, or have had made under my supervision chimeric polypeptides containing an intracellular loop of PAR1 and PAR4 and a several hydrophobic moieties (some of which were fatty acids and some of which were not fatty acids). The activity of the constructs to modulate platelet aggregation was also evaluated.

4. Peptides based on the third intracellular loops of PAR1 and PAR4 were synthesized and coupled at their amino-termini with straight chain fatty acids or steroid acids as described in the specification of the patent application (see attached Figures A-D). MALDI mass spectrometry confirmed that the correct acyl-peptides and steroid-peptides were synthesized (Figure A). The structures of PAR1 and PAR4-based pepducins with N-acyl chains ranging from C8 (caprylate) to C18 (stearate) are shown in Figure B. The structures of PAR1 and PAR4 pepducins with N-terminal steroids (cholate, deoxycholate, chenodeoxycholate, lithocholate, and cholanic) are shown in Figures C-D and their activity as agonists or antagonists of PAR1 and PAR4 are shown in Figures E-I.

5. Figure E shows that the PAR1 i3 loop-based peptide, P1-12 (RCLSSSAVANRS), derivatized with myristate, cholanic, lithocholate, cholate, or laurate creates pepducins that are antagonists of SFLLRN-induced human platelet aggregation. Human platelets were gel-purified and resuspended at 150,000/μL in PIPES buffer supplemented with 2 mM CaCl. Platelet aggregation was assessed as the maximal change in light transmission occurring within fifteen minutes following the addition of the PAR1 agonist 125 nM SFLLRN using a 4-channel chronolog 560VS/490-2D aggregometer. All aggregation measurements were made at 37 °C while stirring at 900 rpm in final volumes of 250 μL. Platelets were preincubated for 10 min with 1 μl DMSO or pepducin dissolved in the same amount of DMSO. Five PAR1 pepducins were tested: P1-12 coupled with myristic acid (P1-myr-12), cholanic acid (P1-CL-12), lithocholic acid (P1-LCA-12), cholic acid (P1-CA-12) or with lauric acid (P1-lar-12). In this assay, the P1-myr-12 pepducin (IC50=0.5 μM) was the most potent antagonist tested against PAR1-dependent human platelet aggregation (Figure G).

6. In Figure F, five PAR4-based P4-10 (SGRRYGHALR) pepducins were tested as inhibitors of human platelet aggregation as shown in Figure E. The platelets were activated with the PAR4 agonist peptide, 200 μM AYPGKF. The steroid-derivatized P4-CL-10 and P4-LCA-10 pepducins were the most potent antagonists (IC50= 0.3 μM) of PAR4-induced platelet aggregation (Figure G). Of the acyl chain-derivatized P4-10 pepducins, the C16 (P4-pal-10) derivative was slightly more potent than the C14 (P4-myr-10) and both were more potent than the C18 (P4-ste-10)-derivatized P4-10 pepducin.

7. Previously, we showed that the longer PAR1 i3 loop pepducin, P1pal-19 and the shorter C-terminal version P1pal-13 were full or partial agonists of PAR1-dependent signaling in recombinant systems and in human platelets. We have now synthesized a series of P1-19 based peptides (RCLSSSAVANRSKKSRALF) that were derivatized at their N-termini with the bile steroids cholate, deoxycholate and cholanic as well as with the acyl lipids caprylate, capric, laurate, myristate, and palmitate (Figure A). All of these were tested as agonists of PAR1-dependent Ca2+ fluxes using the CEM human T-cell based cell line. As shown in Figures H-I, P1-LCA-19 was the most potent agonist (EC50=400 nM) of this series of P1-19-based pepducins. P1-pal-19 was next in potency (EC50=3 μM) followed by P1-myr-19 (EC50=5 μM) and then P1-DCA-19, P1-CA-19, P1-cpr-19 and P1-lar-19.

8. The results of these studies data indicate that the methods described in the specification of the patent application have been used to make a variety of chimeric polypeptides encompassed by the claims. Examples of several intracellular domain portions of GPCR proteins coupled with several different hydrophobic moieties were generated and found to be active. In particular, numerous examples of PAR peptides linked to lipids were found to be effective at modulating the aggregation of platelets. Thus, I believe that the Examiner should withdraw the rejections and allow the pending claims.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, Title 18, United States Code, and that willful false statements may jeopardize the validity of this application and any patent issuing therefrom.

Athan Kuliopulos

Signed at TUFTS-NEMC
this 9 day of January, 2004

Figure A

The values are calculated and (actual) molecular masses (Da) measured by MALDI mass spectrometric analysis

Lipids	Abbrev	Alone	with P4-10 (1171.34)	with P1-12 (1292.42)	with P1-19 (2080)
Cholic acid	CA	408.6		1676 (1677.16)	2471 (2472.44)
Deoxycholic acid	DCA	392.6		1624 (1525.11)	2455 (2456.15)
Chenodeoxycholic acid	CDA	490.7		1722 (1723.15)	
Lithocholic acid	LCA	376.6	1529.9 (1530.79)	1608 (1609.18)	2439 (2440.32)
Cholanic acid	CL	360.6	1513.9 (1514.96)	1592 (1593.28)	
Caprylic acid-C8	cpr	144		1375 (1377.21)	2206 (2206.35)
Capric acid-C10	cap	172		1403 (1405.17)	2234 (2234.04)
Lauric acid-C12	lar	200		1431 (1433.94)	2262 (2262.5)
Myristic acid-C14	myr	228	1381.3 (1382.35)	1459 (1461.71)	2290 (2292.35)
Palmitic acid-C16	pal	256	1409.3 (1408.74)	1487 (1486.82)	2317.86 (2318.87)
Stearic acid-C18	ste	284.5	1437.8 (1439.16)		

Figure B

P1=PAR1
P4=PAR4

cpr=caprylate C8
cap=caprate C10
lar=laurate C12
myr=myristate C14
pal=palmitate C16
ste=stearate C18

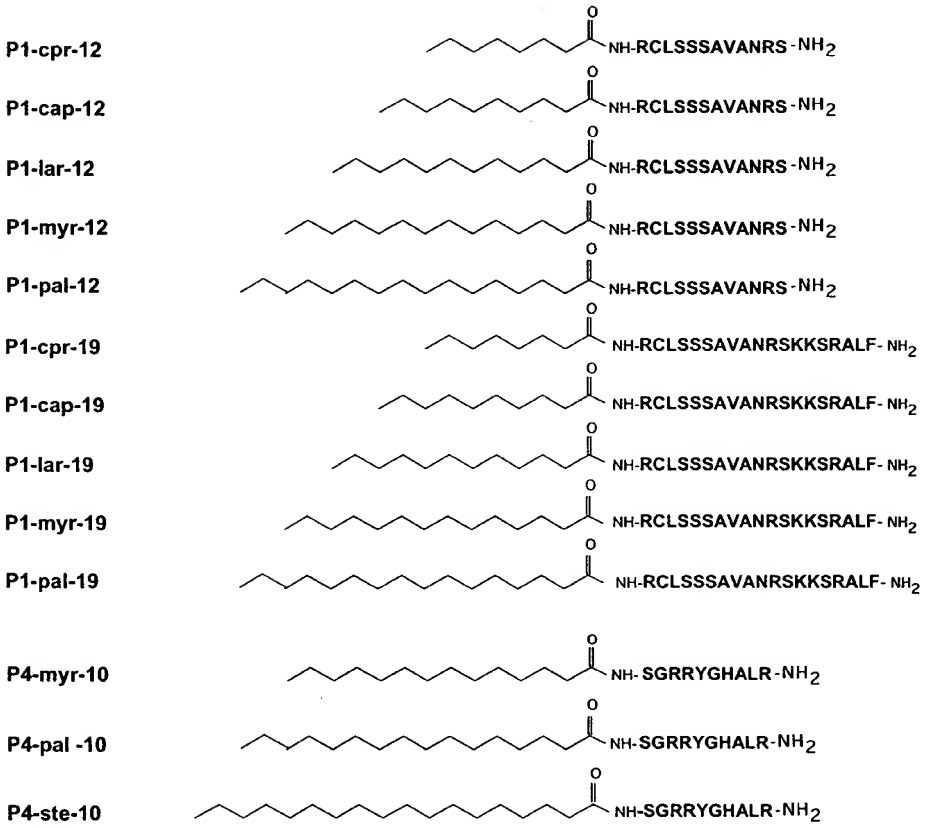
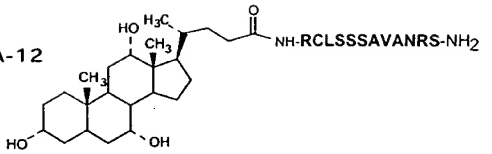


Figure C

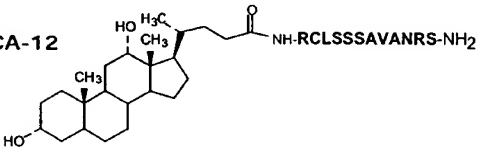
P1=PAR1

CA=Cholate
DCA= Deoxycholate
CDA=Chenodeoxycholate
LCA=Lithocholate
CL=Cholanic

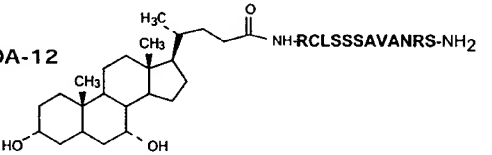
P1-CA-12



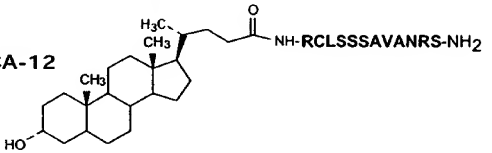
P1-DCA-12



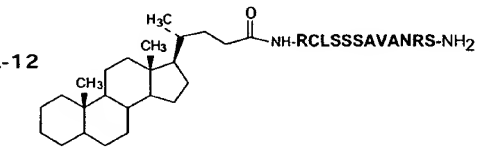
P1-CDA-12



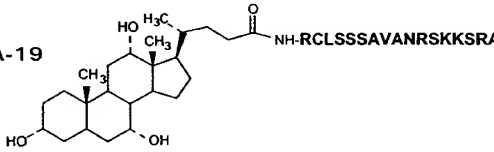
P1-LCA-12



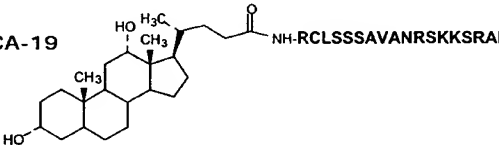
P1-CL-12



P1-CA-19



P1-DCA-19



P1-LCA-19

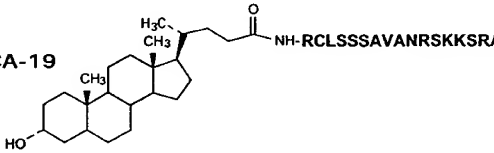


Figure D

P4=PAR4

LCA=Lithocholate
CL=Cholanic

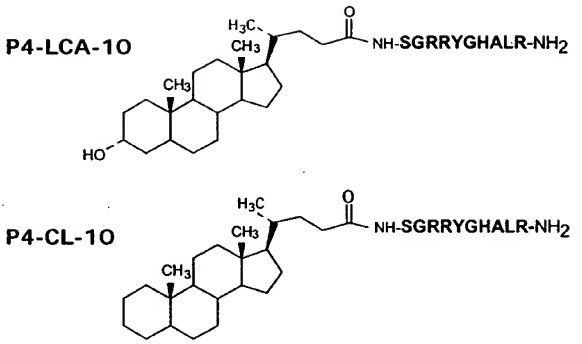


Figure E

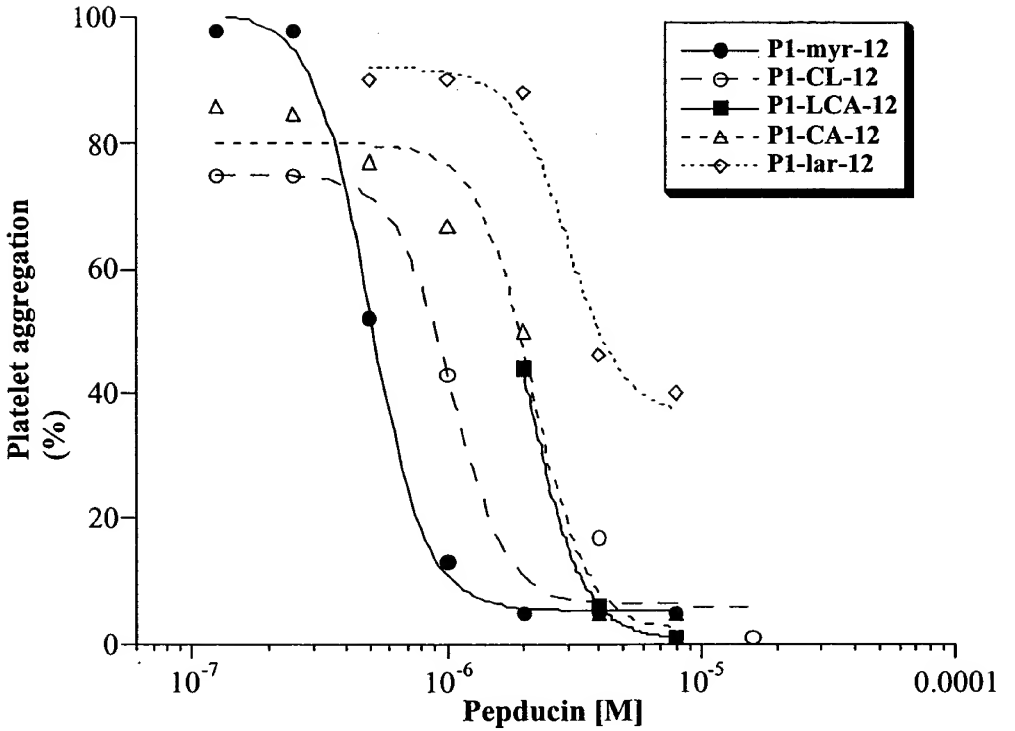

P1-myr-12
P1-CL-12
P1-LCA-12
P1-CA-12
P1-lar-12
Platelet aggregation (%)
100
80
60
40
20
0
10^{-7}
10^{-6}
10^{-5}
0.0001
Pepducin [M]

Figure F

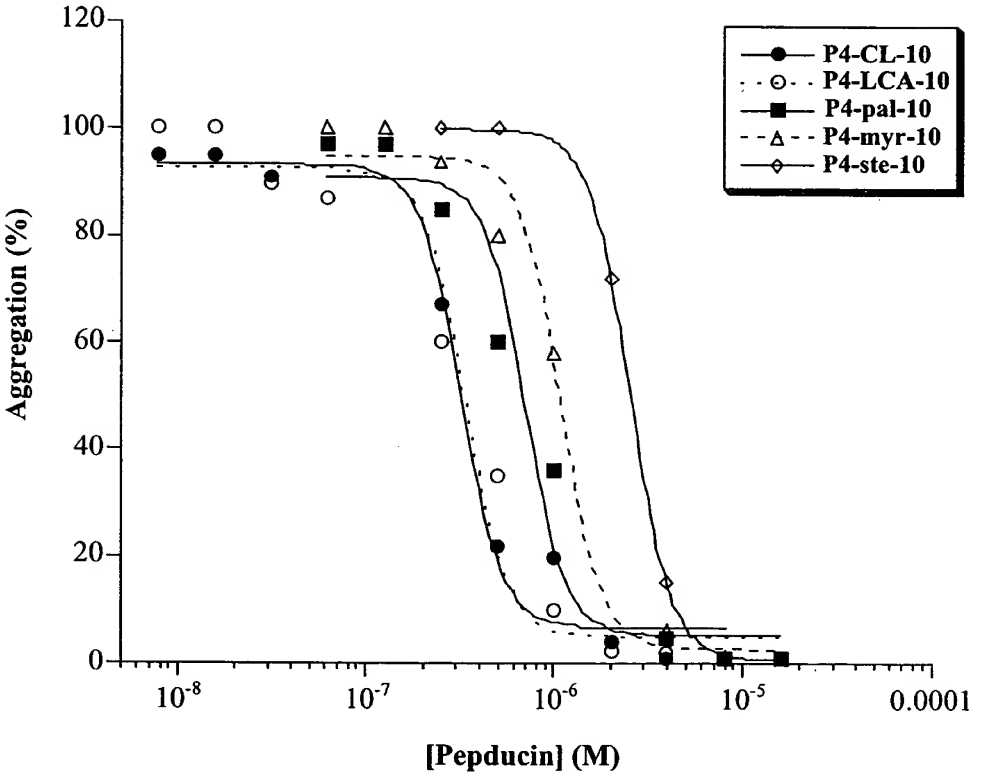

120
100
80
60
40
20
0
Aggregation (%)
P4-CL-10
P4-LCA-10
P4-pal-10
P4-myr-10
P4-ste-10
10-8
10-7
10-6
10-5
0.0001
[Pepducin] (M)

Figure G

Pepducins	IC_{50} for platelet aggregation (microM)
P1-myr-12	0.5
P1-pal-12	1
P1-CL-12	1
P1-LCA-12	1.4
P1-CA-12	2.2
P1-lar -12	3
P4-CL-10	0.3
P4-LCA-10	0.3
P4-pal-10	0.7
P4-myr-10	1.0
P4-ste-10	2.5

P1 series inhibited 125 nanoMolar SFLLRN, except for P1pal-12 which inhibited 3 microMolar SFLLRN
P4 series inhibited 200 microMolar AYPGKF

Figure H

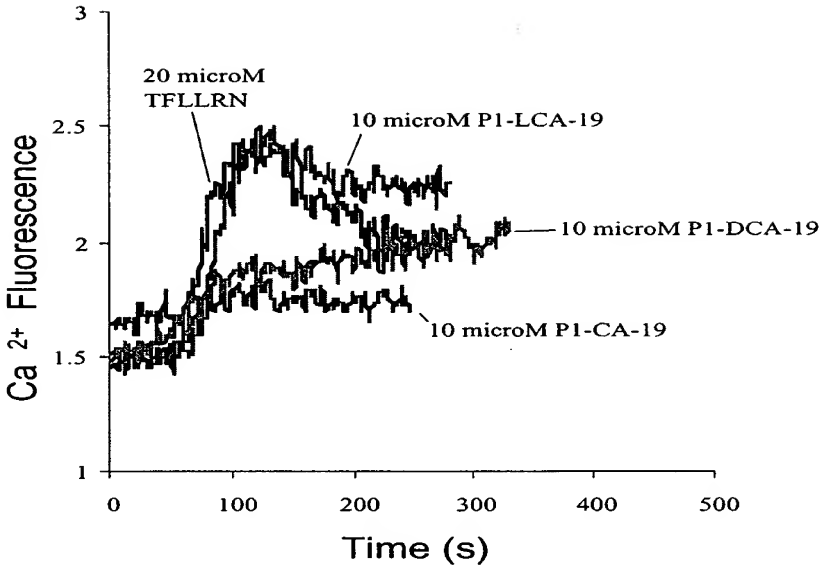
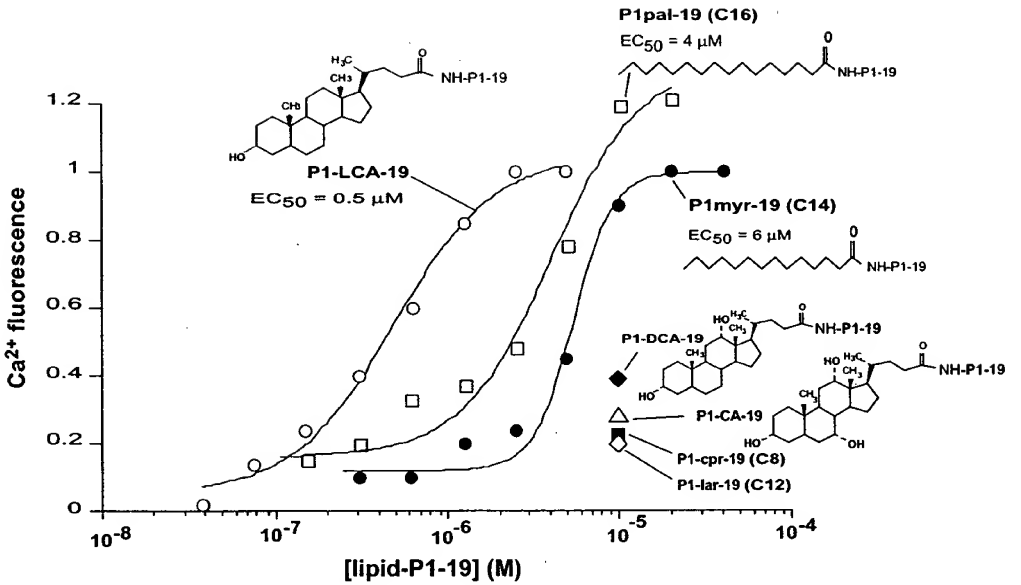

3
2.5
2
1.5
1
Ca 2+ Fluorescence
20 microM
TFLLRN
10 microM P1-LCA-19
10 microM P1-DCA-19
10 microM P1-CA-19
0
100
200
300
400
500
Time (s)

Figure I


P1pal-19 (C16)
EC50 = 4 μM
NH-P1-19
P1-LCA-19
EC50 = 0.5 μM
P1myr-19 (C14)
EC50 = 6 μM
P1-DCA-19
P1-CA-19
P1-cpr-19 (C8)
P1-lar-19 (C12)
Ca2+ fluorescence
[lipid-P1-19] (M)
1.2
1
0.8
0.6
0.4
0.2
0
10-8
10-7
10-6
10-5
10-4